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Human Immunodeficiency Virus Reactivation by Phorbol Esters or T-Cell Receptor Ligation Requires both PKCα and PKCθ

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Latently human immunodeficiency virus (HIV)-infected memory CD4$^+$ T cells represent the major obstacle to eradicating HIV from infected patients. Antigens, T-cell receptor (TCR) ligation, and phorbol esters can reactivate HIV from latency in a protein kinase C (PKC)-dependent manner; however, it is unknown which specific PKC isoforms are required for this effect. We demonstrate that constitutively active (CA) forms of both PKCθ, PKCθA148E, and PKCα, PKCαA25E, induce HIV long terminal repeat (LTR)-dependent transcription in Jurkat and primary human CD4$^+$ T cells and that both PKCθA148E and PKCαA25E cause HIV reactivation in J1.1 T cells. Suppression of both PKCα and PKCθ with short hairpinned (sh) RNA inhibited CD3/CD28-induced HIV LTR-dependent transcription and HIV reactivation in J1.1 T cells. Both prostratin and phorbol myristate 13-acetate induced HIV LTR-dependent transcription and HIV reactivation in J1.1 T cells that was blocked by shRNA against either PKCα or PKCθ. Since suppression of PKCα and PKCθ together has no greater inhibitory effect on HIV reactivation than inhibition of PKCα alone, our data confirm that PKCα and PKCθ act in sequence. The requirement for PKCα and PKCθ for prostratin-induced HIV reactivation and the ability of selective PKCα or PKCθ agonists to induce HIV transcription indicate that these PKC isoforms are important targets for therapeutic drug design.

The effectiveness of current antiretroviral agents is underscored by their inability to impact transcriptionally inactive viral reservoirs. Accordingly, human immunodeficiency virus (HIV) viral rebound is commonly observed when patients cease taking antiretroviral agents (15, 18, 19, 50, 58). Sequence analysis of rebound viral isolates indicates significant homology with proviral sequences from infected memory CD4$^+$ T cells (61), suggesting that such cell populations act as a principal viral reservoir that is unaffected by antiretroviral therapy. Indeed, memory CD4$^+$ T cells which contain HIV provirus have been defined to be transcriptionally inactive and to have an in vivo half-life of several years (13, 14). Consequently an estimated 60 or more years of fully supportive antiretroviral therapy may be required to fully purge such a reservoir, a time frame which is both impractical and clinically not useful. Three conceptually distinct approaches have been proposed as ways of impacting such viral reservoirs: toxins designed to eliminate virus-containing cells (5, 21, 38), antiviral intensification strategies to prevent repopulation of the latent pools (12, 26, 32, 37, 59), and compounds which stimulate HIV long terminal repeat (LTR) transcription, thereby rendering such cells susceptible to antiretroviral agents and/or the cytotoxic effects of viral replication (30, 49).

The HIV type 1 (HIV-1) LTR is regulated in large part by cellular transcription factors, including NF-κB, AP-1, SP-1, and NF-AT (reviewed in reference 28). Consequently, stimuli which activate these transcription factors cause HIV LTR-driven viral replication (17, 20, 41). Antigen presentation or T-cell receptor (TCR) cross-linking can induce HIV reactivation from latently infected CD4$^+$ T cells in vitro (15) through protein kinase C (PKC)-mediated activation of NF-κB (40). Direct activation of PKC by phorbol esters reactivates HIV from latency in cell lines (27, 29, 45) and, more importantly, it induces HIV reactivation in peripheral blood mononuclear cells and primary quiescent T cells (1, 9, 10). PKC activation induces HIV from latency by targeting multiple regulatory elements of HIV LTR. In addition to NF-κB, PKC induces HIV LTR transcription via activation of the AP-1 transcription factor; however, its action is largely dependent on a cooperation with NF-κB (35, 46, 60). PKC also increases HIV-1 gene expression by phosphorylation of the virally encoded TAT transcription factor and cellular TAR-binding factors (25, 27).

Among the PKC isoforms in T cells, the novel PKC isoform PKCθ plays a crucial role in T-cell activation and proliferation (3, 6, 51). PKCθ activates NF-κB (16, 36, 51) and AP-1 (4, 56) transcription factors, which bind to multiple sites in the enhancer region of the HIV-1 LTR. In addition PKCα acts upstream of PKCθ to induce NF-κB following CD3/CD28 ligation (54). Since HIV reactivation following CD3/CD28 ligation is NF-κB dependent, together these data suggest that each of these two PKC isoforms may be required to mediate reactivation of latent HIV. Indeed, inhibition of both conventional and novel PKC isoforms blocks HIV reactivation following costimulation with CD3 and CD28 antibodies in the SCID-hu(Thy/Liv) model (9).

While TCR cross-linking can reactivate latent HIV in primary T cells in vitro (9, 11), clinical efforts to reduce the latent pool of HIV by treatment with the anti-CD3 antibody OKT-3 alone or in combination with interleukin-2 fails to meaningfully decrease the viral reservoir (32, 44, 55). The phorbol

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esters (8, 33) and 1,2-diacylglycerol analogs (24), including the non-tumor-promoting phorbol ester prostratin (23), have been suggested as agents to reactivate HIV and eradicate the pool of latently HIV-infected $CD4^+$ T cells (22, 23). More recently, phorbol esters including prostratin have been directly evaluated for their ability to reactivate latent virus (30, 31). Indeed, prostratin causes significant reactivation of latent HIV from memory T-cell pools (7, 10, 30, 48). The mechanism by which prostratin causes viral reactivation is, therefore, of interest, and two independent studies now suggest that conventional and novel PKC isoforms are required (22, 57), although which specific PKC isoforms are involved and whether they are absolutely required is unknown. The present study was designed to address the specific requirement of different PKC isoforms following TCR- or phorbol ester-mediated HIV LTR reactivation and to determine whether such PKC isoforms are absolutely required.

MATERIALS AND METHODS

Plasmids. Generation and characterization of isoform-specific PKC suppression vectors were previously described (54). The HIV LTR and HIV LTR (ΔκB)-dependent firefly luciferase reporter expression vector (κB-luc) has been previously described (17). The pRL-TK expression vector, which provides constitutive expression of *Renilla* luciferase, is commercially available (Promega, Madison, WI). GFP-PCI was a gift from D. McKean (Mayo Clinic, Rochester, MN). The Sp1-luciferase reporter was kindly provided by D. Billadeau (Mayo Clinic, Rochester, MN). The mammalian expression vector, pEF1/*Myc*-His, was purchased from Invitrogen (Carlsbad, CA). Constitutively active forms, PKCαA25E and PKCθA148E, were previously described (54).

Constitutively active IKKβS177E/S181E was generated by site-directed mutagenesis using mutagenic primers (sense, 5′-GAACTTTGCACAGAATTCGT GGGGA; antisense, 5′-TTCTGTGCAAAGTTCGCCCTGATCC; sense IKKβ with BamHI site [underlined], GGGATCCATCCATGAGCTGGTCACCT; antisense IKKβ with XbaI site [underlined], CGTCTAGATTATGAGGCCTGCT CCAG) and cloned into pGEM-T Easy. The cDNA coding for IKKβS177E/S181E was excised and subcloned into HA-pcDNA3 I at the BamHI XbaI site. The sequences of all generated constructs were verified by sequencing.

Cell culture and reagents. Jurkat T cells and latently HIV-1-infected J1.1 T cells were obtained from the American Type Culture Collection, Rockville, MD and maintained in RPMI 1640 (BioWhittaker, Walkersville, MD) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin-streptomycin, and 2 mM L-glutamine. Cells were grown to a density of 3×10^5 to 5×10^5/ml at the time of the different experiments. Ionomycin was purchased from Calbiochem (La Jolla, CA), and tumor necrosis factor alpha was purchased from R&D Systems (Minneapolis, MN). Leupeptin, aprotinin, and pepstatin A were obtained from Boehringer Mannheim (Indianapolis, IN). Antihemagglutinin (anti-HA) high-affinity antibodies were purchased from Boehringer Mannheim (Indianapolis, IN). OKT3 antibodies were obtained from Ortho Biotech (Raritan, NJ), and anti-CD28 was purchased from BD Biosciences (San Jose, CA). Anti-PKCα (C-20) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-PKCβ1 and anti-PKCθ were purchased from BD Transduction Laboratories (San Diego, CA).

To isolate $CD4^+$ T cells, peripheral blood mononuclear cells from healthy volunteer blood donors were obtained from buffy coats by using RosetteSep cocktail $CD4^+$ antibodies in accordance with the manufacturer's protocol (StemCell Technologies, Vancouver, British Columbia, Canada). The remaining cell population was repeatedly found to be 98% $CD4^+$ T cells as determined by flow cytometry. $CD4^+$ T cells used in the various experiments were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA), 2 mM L-glutamine, and antibiotics (penicillin [100 U/ml] and streptomycin [100 μg/ml]) at 0.5×10^6 cells/ml. For Jurkat T cells, ionomycin was used at 3.5 μg/ml. Jurkat and J1.1 T cells were cross-linked with 10 μg/ml of anti-CD3 and anti-CD28 antibodies or isotype control antibodies as previously described (53).

Cell extract preparation and immunoblotting. To obtain total cellular proteins, cells were washed with cold phosphate-buffered saline, resuspended in a modified whole-cell extract PD buffer (53) (40 mM Tris-HCl [pH 8], 0.3 M NaCl, 0.1% Nonidet P-40, 6 mM EDTA, 6 mM EGTA, 10 mM NaF, 10 mM *P*-nitrophenyl phosphate 10 mM β-glycerophosphate, 300 μM sodium orthovanadate, 1 mM dithiothreitol, 2 μM phenylmethylsulfonyl fluoride, aprotinin at 10 μg/ml, leupeptin at 1 μg/ml, pepstatin 1 μg/ml) and centrifuged at $12{,}000 \times g$ for 15 min at 4°C. The resulting supernatant contained total cellular protein. The amount of cellular protein present in the clarified supernatant was calculated by using the Bio-Rad (Hercules, CA) protein assay. For Western immunoblot assays, equal amounts of whole-cell extract were loaded and separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to Immobilon-P membranes (Millipore, Bedford, MA). Immunoblotting was performed with specific antibodies and visualized by using the ECL Western blotting detection kit (Amersham, Buckinghamshire, England).

Gene transfection, p24 enzyme-linked immunosorbent assay (ELISA), and reporter assays. Jurkat T and J1.1 T cells were electroporated using a BTX Electro Square Porator T820 (BTX Corporation, San Diego, CA) at 325 V, 10 ms, and 350 V, 10 ms, correspondingly. Primary $CD4^+$ T cells were electroporated by using Nucleofector (U-14 program; Amaxa Inc., Gaithersburg, MD) in accordance with a protocol for unstimulated human T cells. Where indicated, transfection efficiency was monitored by green fluorescent protein (GFP) expression.

Jurkat T cells were transfected with the indicated plasmids and grown for 18 to 24 h. Cells were stimulated for 12 h with ionomycin (3.5 μg/ml), prostratin (10 μM), or phorbol myristate 13-acetate (PMA; 10 ng/ml) or cross-linked with anti-CD3 (10 μg/ml) and anti-CD28 (10 μg/ml) antibodies as previously described (54). Thereafter, cells were washed twice in cold phosphate-buffered saline and lysed with 100 μl lysis buffer (Dual-Luciferase reporter assay system; Promega, Madison, WI). Firefly and *Renilla* luciferase activities from 20 μl of extract were assayed using the Promega Dual-Luciferase reporter assay system reagents and a Berthold Lumat following the manufacturer's recommendations. κB-Luciferase activity was normalized to *Renilla* expression. All transfection experiments were performed in duplicate.

Concentrations of p24 Gag in supernatants of J1.1 T cells were measured in duplicate by using a p24 ELISA kit (ZeptoMetrix Corporation, Buffalo, NY).

RESULTS

Constitutively active forms of both PKCα and PKCθ induce HIV LTR activation via NF-κB in Jurkat and human primary $CD4^+$ T cells. Recently we demonstrated that PKCα acts upstream of PKCθ to activate NF-κB following CD3/CD28 stimulation in uninfected T cells (54). Since antigen presentation, TCR ligation, and phorbol esters all lead to NF-κB activation and subsequent HIV LTR transcription, we first examined the role of both PKCα and PKCθ in induction of HIV-1 LTR transcriptional activity in T lymphocytes mediated by these stimuli.

First, we studied whether CA forms of PKCα, PKCαA25E, and PKCθ, PKCθA148E, can induce HIV-1 LTR transcriptional activity. Jurkat T cells were transfected with either PKCαA25E or PKCθA148E, together with luciferase reporter plasmids containing the wild-type HIV-1 LTR or the LTR lacking the two κB sites, HIV-LTR (ΔκB). As shown in Fig. 1A, expression of either PKCαA25E or PKCθA148E induced HIV LTR transcriptional activity preferentially via NF-κB, since deletion of κB sites strongly impaired HIV LTR activation (Fig. 1A). As previously shown (54), ionomycin treatment is required for efficient NF-κB activation by PKCαA25E, since expression of PKCαA25E resulted in only a moderate increase of HIV LTR transcriptional activity (Fig. 1A) which was further augmented by ionomycin treatment. Similar to PKCθA148E, PKCαA25E induced HIV LTR transcription via κB sites (Fig. 1A). While PKCαA25E and PKCθA148E strongly activated NF-κB-dependent transcription (25- to 200-fold) of HIV LTR, both only modestly induced (1.2- to 2-fold) transcriptional activity of the transcription factor, Sp1 (Fig. 1B).

To further confirm the relevance of these findings, we stud-

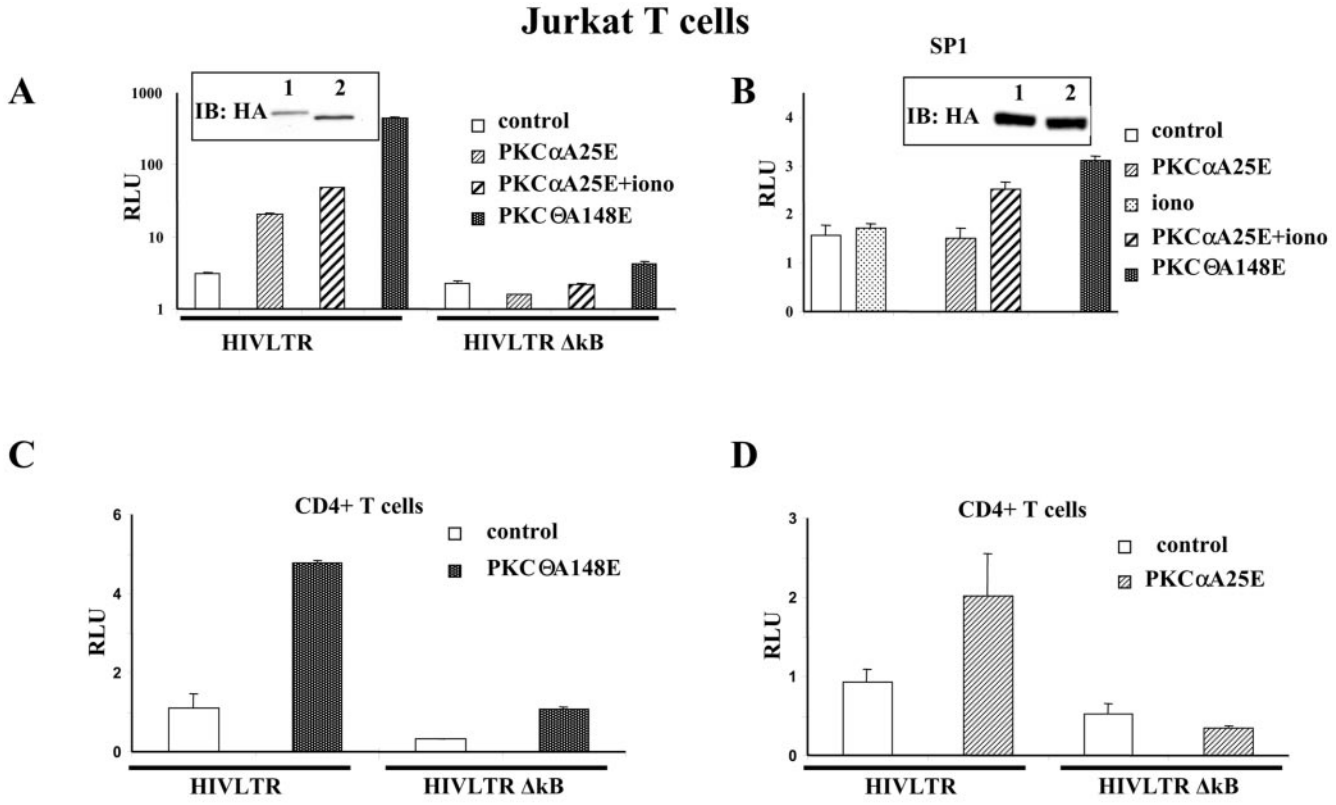

FIG. 1. Constitutively active forms of both PKCα and PKCθ induce HIV LTR activation via the κB site in Jurkat and human primary $CD4^+$ T cells. (A) Jurkat T cells (10×10^6 per sample) were electroporated with 20 μg of catalytically active PKCα(pEF-BOS/HA-PKCαA25E), catalytically active PKCθ(pEF-BOS/HA-PKCθA148E), or control vector pEF-BOS together with either HIV LTR-luciferase reporter (3.8 μg) or HIV LTR (ΔκB)-luciferase reporter (3.8 μg) and *Renilla* luciferase reporter (0.2 μg) plasmids. Twenty hours later, HA-PKCαA25E-transfected Jurkat T cells were treated with 3.5 μg/ml of ionomycin. Four hours later cells were harvested and luciferase activities were measured. The HIV LTR-dependent luciferase activity was normalized to *Renilla* luciferase activity (relative luciferase activity). All experiments were performed in duplicate. Expression of HA-PKCαA25E (lane 1) and HA-PKCθA148E (lane 2) was analyzed by immunoblotting with anti-HA antibodies (IB: HA). (B) Same experiment as in panel A, except Sp1-luciferase reporter was used. (C and D) Freshly isolated human primary $CD4^+$ T cells (5×10^6 per sample) from healthy donors were nucleofected (Nucleofector; U-14 program) with 2 μg of either HA-PKCαA25E, HA-PKCθA148E, or control vector together with either HIV LTR or HIV LTR (ΔκB)-luciferase reporter (1.8 μg) and *Renilla* luciferase reporter (0.2 μg) plasmids. Eighteen hours later, $CD4^+$ T cells were harvested and luciferase activity was measured.

ied whether PKCαA25E and PKCθA148E can induce HIV-1 LTR transcriptional activity in human primary $CD4^+$ T cells. Purified human $CD4^+$ T cells were transfected with either PKCαA25E or PKCθA148E, together with the luciferase reporter plasmids mentioned above. Similar to our results with Jurkat T cells, expression of either PKCαA25E or PKCθA148E in human primary $CD4^+$ T cells induced HIV-LTR transcriptional activity via κB sites (Fig. 1C and D).

Taken together these results demonstrate that both PKCα and PKCθ can induce HIV LTR transcription preferentially through κB sites in Jurkat and primary $CD4^+$ T cells.

Suppression of either PKCα or PKCθ inhibits HIV LTR activation by CD3/CD28 stimulation in Jurkat T cells. To identify which of the two PKC isoforms mediates CD3/CD28-induced HIV LTR activation, we utilized isoform-specific PKC suppression vectors (54), which direct synthesis of short hairpinned (sh) RNA molecules specific for targeting of either human PKCα or human PKCθ. Jurkat T cells were transfected with either pFRT-PKCα or pFRT-PKCθ together with luciferase reporter plasmids as mentioned above. The levels of PKCα and PKCθ expression in transfected cells were analyzed 48 h later by immunoblotting with anti-PKCα and anti-PKCθ antibodies. As shown in Fig. 2C, transfection of Jurkat T cells with either pFRT-PKCα or pFRT-PKCθ resulted in selective and significant reduction of PKCα and PKCθ expression. Neither of the shRNA constructs affected the levels of PKCβ1. Cross-linking of transfected Jurkat T cells with CD3 and CD28 antibodies induced HIV LTR-dependent luciferase activity preferentially via κB sites of LTR (Fig. 2A and B). Suppression of either PKCα or PKCθ significantly impaired CD3/CD28-induced HIV LTR-dependent luciferase activity. In contrast, suppression of either PKCα or PKCθ had no effect on a basal or prostratin-induced Sp1-dependent luciferase activity (Fig. 2D), suggesting that both PKCs are dispensable for Sp1 transcription. Altogether, these results indicate that both PKC isoforms are required for CD3/CD28-induced HIV LTR-dependent transcription via κB sites in Jurkat T cells.

Constitutively active forms of both PKCα and PKCθ reactivate HIV in latently infected J1.1 T cells. To further define the role of both PKCs in HIV reactivation, we studied HIV LTR-dependent transcription and HIV-1 production in the latently HIV-infected $CD3^+$ J 1.1 T-cell line (42). J1.1 T cells

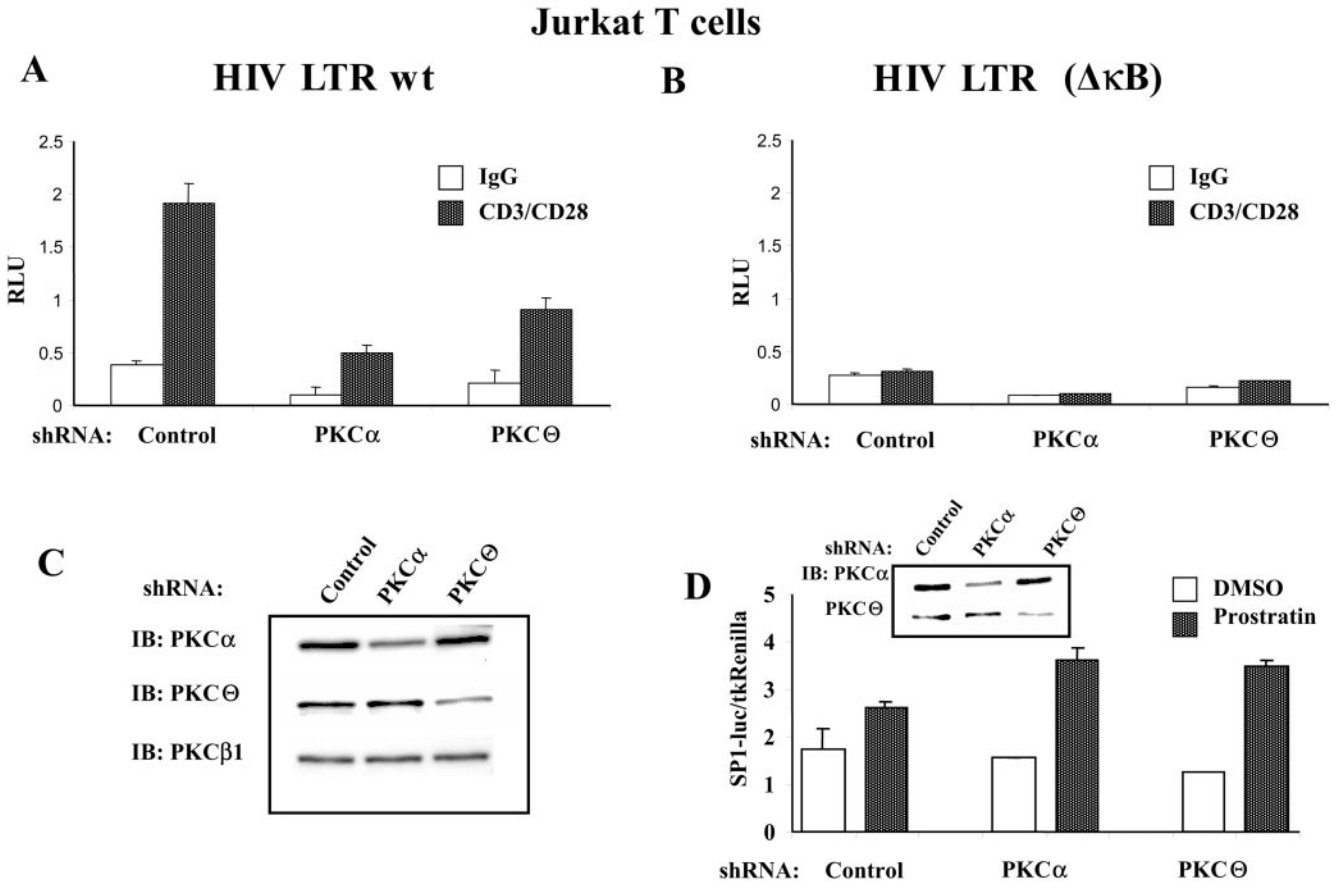

FIG. 2. PKCα and PKCθ are required for CD3/CD28-induced HIV LTR-dependent transcriptional activity in Jurkat T cells. (A and B) Jurkat T cells (10×10^6 per sample) were electroporated with 40 μg of pFRT-PKCα or pFRT-PKCθ or with pFRT control vector together with either HIV LTR-luciferase reporter (3.8 μg) (A) or HIV-LTR (ΔκB)-luciferase reporter (3.8 μg) (B) and *Renilla* luciferase reporter (0.2 μg) plasmids. Forty-eight hours later, the transfected cells were treated with anti-CD3 (10 μg/ml) and anti-CD28 (10 μg/ml) antibodies for 45 min on ice and cross-linked on plated goat anti-mouse antibodies at 37°C. Four hours later, cells were harvested and luciferase activity was measured. (C) Protein levels following the suppression of endogenous PKCα and PKCθ expression was confirmed by immunoblotting. Equal amounts of protein per lane were demonstrated by immunoblotting with anti-PKCβ1 antibodies. (D) Same experiment as in panel A, except Sp1-luciferase reporter and prostratin at 10 μM were used.

were transfected with HA-PKCαA25E or HA-PKCθA148E or, as a positive control, with the constitutively active form of IKKβ, HA-IKKβS177E,S181E, together with either HIV LTR (Fig. 3A) or HIV LTR (ΔκB)- luciferase reporters (Fig. 3B) and the tk*Renilla* reporter plasmid. Thirty-six hours later, supernatants were collected to measure p24 Gag levels and cells were harvested to assay luciferase activities. Expression of either PKCθA148E or PKCαA25E induced HIV LTR transcriptional activity in J1.1 T cells through both NF-κB-dependent and non-NF-κB-dependent pathways (Fig. 3A and B).

To test whether PKCαA25E, HA-PKCθA148E, or HA-IKKβS177E,S181E can reactivate HIV from latency, we measured p24 Gag levels in supernatants of transfected J1.1 cells. As shown in Fig. 3D, the expression of the constitutively active forms of PKCα, PKCθ, or IKKβ induce strong p24 Gag production in J1.1 T cells, which correlates with HIV LTR activation (Fig. 3A and B).

Taken together, these results demonstrate that activation of either PKCα or PKCθ is sufficient for the activation of HIV LTR transcription and for subsequent HIV reactivation in latently infected T cells.

Suppression of either PKCα or PKCθ inhibits HIV reactivation by CD3/CD28 stimulation in J1.1 T cells. To confirm which of the two PKC isoforms is required for the CD3/CD28-induced HIV reactivation, J1.1 T cells were transfected with control vector, pFRT-PKCα alone, pFRT-PKCθ alone, or pFRT-PKCα together with pFRT-PKCθ. HIV LTR or HIV LTR (ΔκB) luciferase plasmids and *Renilla* were cotransfected. Forty-eight hours later cells were cross-linked with anti-CD3 and anti-CD28 antibodies and then cells and supernatants were harvested to assay luciferase activity (Fig. 4A), PKC expression (Fig. 4B), and p24 Gag concentrations (Fig. 4C). As shown in Fig. 4A, stimulation of J1.1 T cells with anti-CD3 anti-CD28 antibodies induced a threefold increase in HIV LTR transcription. Deletion of two κB sites in the LTR resulted in a 50% decrease of CD3/CD28-induced HIV LTR transcription. Suppression of PKCα inhibited κB-specific CD3/CD28-induced HIV LTR transcription and resulted in a two-

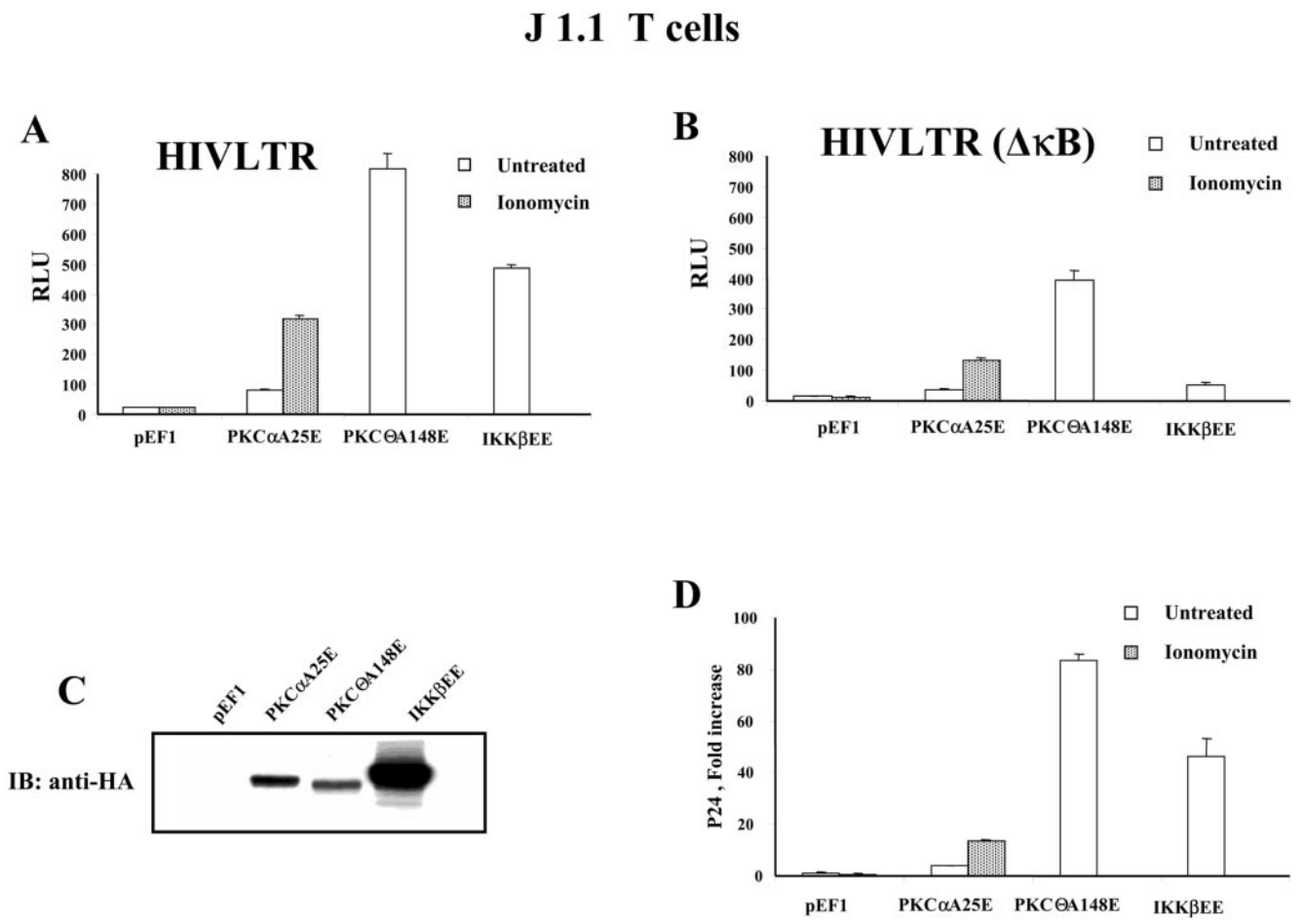

FIG. 3. Constitutively active forms of both PKCα and PKCθ can reactivate HIV in latently infected J1.1 T cells. (A) J1.1 T cells (10×10^6 per sample) were electroporated with 20 μg of catalytically active PKCα(pEF-BOS/HA-PKCαA25E), catalytically active PKCθ(pEF-BOS/HA-PKCθA148E), catalytically active IKKβ(HA-IKKβS177E,S181E), or control vector pEF-BOS together with either HIV LTR-luciferase reporter (3.8 μg) or *Renilla* luciferase reporter (0.2 μg) plasmids. Twenty hours later, HA-PKCαA25E-transfected J1.1 T cells were treated with 3.5 μg/ml of ionomycin. Twelve hours later cells were harvested and luciferase activities were measured. The HIV LTR-dependent luciferase activity was normalized to *Renilla* luciferase activity (RLU). (B) Same experiment as in panel A except that the HIV LTR (ΔκB)-luciferase reporter plasmid was transfected. (C) Expression of HA-PKCαA25E, HA-PKCθA148E, and HA-IKKβS177E,S181E was demonstrated by immunoblotting with anti-HA antibodies (IB:HA). (D) Levels of p24 from supernatants of J1.1 cells transfected as described for panel A were measured by using a p24 ELISA. p24 concentrations (in ng/ml) were normalized to *Renilla* luciferase light units and are expressed as the fold increase of p24 concentrations from effector plasmid-transfected samples to control vector-transfected cells. All experiments were performed in duplicate.

fold decrease of p24 levels in CD3/CD28-treated J1.1 T cells. Conversely, PKCθ suppression inhibited both κB-dependent and to a lesser degree κB-independent CD3/CD28-induced HIV LTR transcription, potentially altering AP-1 (4, 56) and NF-AT (2, 43, 56) activation. Simultaneous suppression of both PKCs did not have a greater effect on CD3/CD28-induced HIV LTR transcription and p24 production than suppression of PKCα or PKCθ alone (Fig. 4A and C), confirming that PKCα and PKCθ act in sequence following CD3/CD28 stimulation (54).

Both PKCα and PKCθ are required for HIV reactivation by phorbol esters in J 1.1 T cells. Having shown that both PKC isoforms are required for CD3/CD28-mediated HIV reactivation, we addressed the role of both PKCs in phorbol ester-mediated HIV reactivation from latency. Isoform-specific PKC suppression vectors were expressed in J1.1. T cells that were then stimulated with two different phorbol esters, PMA or prostratin. Stimulation of J1.1 T cells with PMA or prostratin induced 12-fold increases in HIV LTR-dependent luciferase activity that was reduced by deletion of the κB sites of HIV LTR [Fig. 5A, HIV-LTR (ΔκB)] or suppression of PKCα. PKCα suppression resulted in a twofold decrease of p24 Gag production induced by PMA or prostratin (Fig. 5B). It also blocked 50% of HIV LTR-dependent transcription induced by either PMA or prostratin. As with CD3/CD28 stimulation, suppression of PKCα had no effect on PMA- or prostratin-induced HIV LTR (ΔκB) transcription (Fig. 5A). Suppression of PKCθ blocked almost 50% of prostratin PMA-mediated HIV LTR activation via κB sites and resulted in a twofold decrease of prostratin- or PMA-induced HIV LTR (ΔκB) transcription. PKCθ suppression also significantly decreased p24 production induced by prostratin ($P < 0.05$) (Fig. 5B). Similar to CD3/CD28 stimulation, suppression of both PKCθ and PKCα together was no more effective in blocking PMA- or

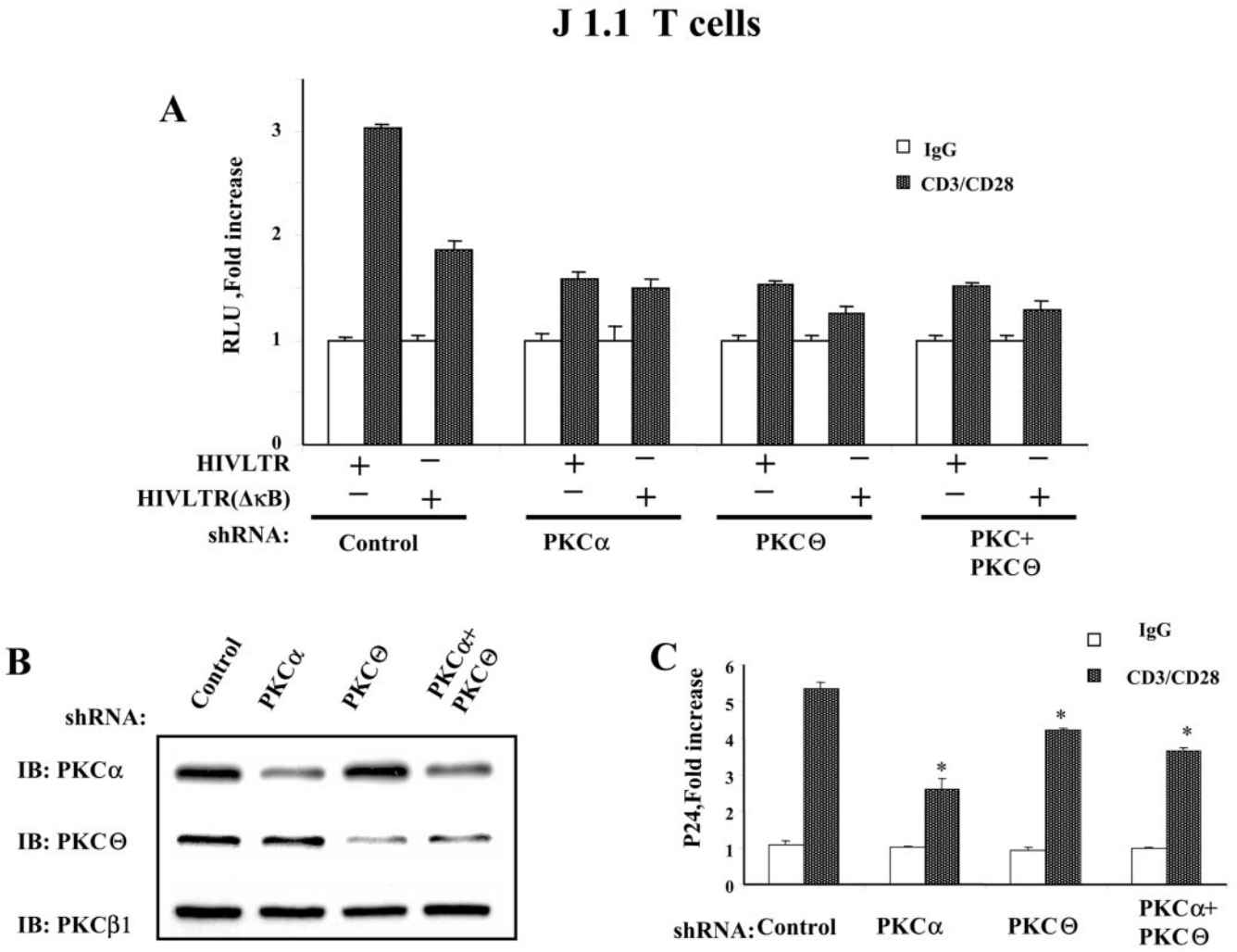

FIG. 4. Suppression of either PKCα or PKCθ inhibits HIV LTR-dependent transcription and HIV reactivation by CD3/CD28 stimulation in J1.1 T cells. (A) Induction of HIV LTR and HIV LTR (ΔκB) transcription by CD3/CD28 cross-linking in latently infected J1.1 T cells is required both PKCα and PKCθ. J1.1T cells (10×10^6 per sample) were electroporated with 30 μg of pFRT-PKCα and 30 μg of pFRT control vector, or 30 μg of pFRT-PKCθ and 30 μg of pFRT control vector, or with 30 μg of pFRT-PKCα and 30 μg of pFRT-PKCθ and 60 μg of pFRT control vector. All transfections were performed together with either HIV LTR-luciferase reporter (1.8 μg) or HIV LTR (ΔκB)-luciferase reporter (1.8 μg) together with *Renilla* luciferase reporter (0.2 μg) plasmids and GFP-expressing vector (5 μg) to control for transfection efficiency and cell viability. Transfection efficiency in all transfections was greater then 65%, and cell viability was higher then 78%. Forty-eight hours later, an equal number of transfected cells (1×10^6 per sample) were treated with anti-CD3 (10 μg/ml) and anti- CD28 (10 μg/ml) antibodies for 45 min on ice and cross-linked on plated goat anti-mouse antibodies at 37°C. Twelve hours later, cells and supernatants were harvested and luciferase activities were measured. The HIV LTR-dependent luciferase activity was normalized to *Renilla* luciferase activity (RLU). Protein levels following the suppression of endogenous PKCα and PKCθ expression were confirmed by immunoblotting with anti-PKCα and anti-PKCθ antibodies. Equal amounts of protein per lane were demonstrated by immunoblotting with anti-PKCβ antibodies (B). (C) p24 data expressed as fold increase of p24 concentrations from CD3/CD28-stimulated samples to immunoglobulin G control-treated samples. Levels of p24 from supernatants of J1.1 cells were measured by using a p24 ELISA. p24 concentrations (in ng/ml) were normalized to *Renilla* luciferase light units. *, $P < 0.05$, for significant difference compared to the pFRT control vector-transfected cells, determined by Student's paired *t* test. All experiments were performed at least three times in duplicate.

prostratin-induced HIV LTR transcription and p24 production than suppression of PKCα alone.

DISCUSSION

In this study we have demonstrated a specific requirement for both PKCα and PKCθ in HIV reactivation following TCR ligation or stimulation with the non-tumor-promoting ester prostratin in T lymphocytes. We conclude this based on observations that selective suppression of PKCα, PKCθ, or both PKC isoforms inhibits HIV LTR transcription and HIV reactivation following CD3/CD28 cross-linking or prostratin treatment in Jurkat and J1.1 T cells. Several interesting features of HIV reactivation by these stimuli are underscored by our results. First, HIV reactivation by either CD3/CD28 engagement or prostratin stimulation requires both PKCα and PKCθ, demonstrating that both PKC isoforms are necessary. Second, suppression of PKCα alone or PKCθ alone has the same inhibitory

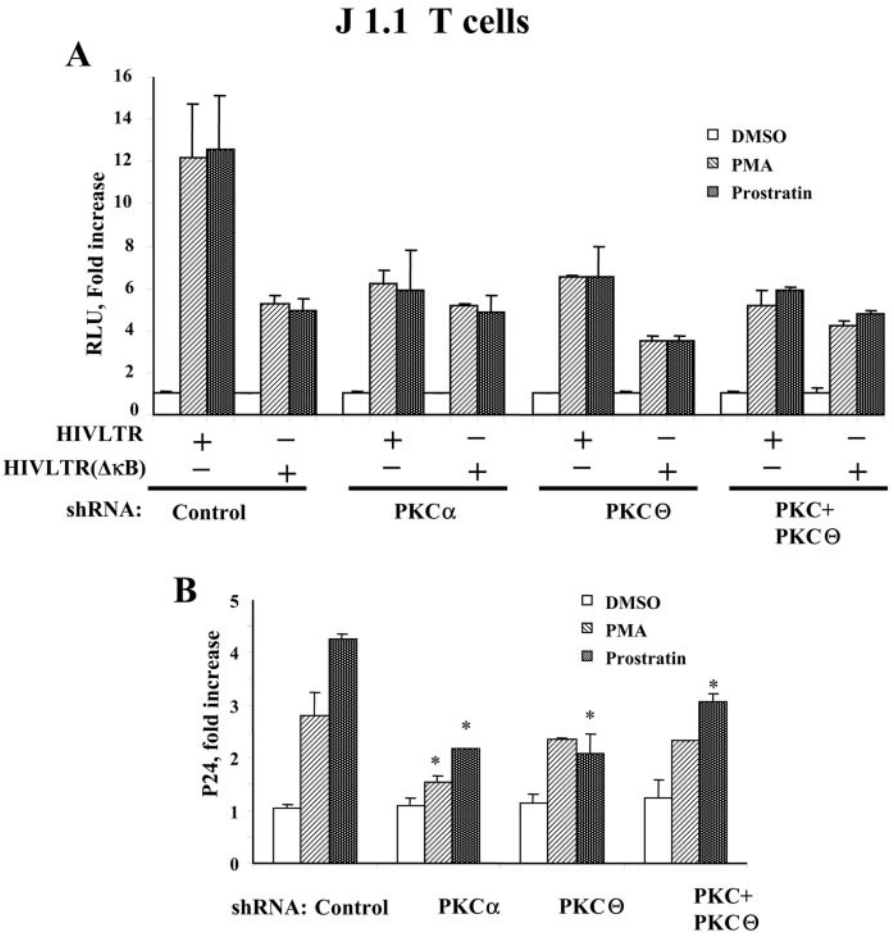

FIG. 5. PKCα and PKCθ cooperate to reactivate HIV following PMA and prostratin stimulation of latently HIV-1-infected J1.1 T cells. (A) PKCα and PKCθ are required for the induction of HIV LTR and HIV LTR (ΔκB) transcription by PMA and prostratin in latently infected J1.1 T cells. J1.1T cells (10×10^6 per sample) were electroporated as described in the legend for Fig. 4. All transfections were performed together with either HIV LTR-luciferase reporter (1.8 μg) or HIV LTR (ΔκB)-luciferase reporter (1.8 μg) together with *Renilla* luciferase reporter (0.2 μg) plasmids and GFP-expressing vector (5 μg) to control for transfection efficiency and cell viability. Cell viability in all transfections was higher then 79%. Transfection efficiency for the pFRT control vector was 66%, for pFRT-PKCα it was 80%, for pFRT-PKCθ it was 74%, and for pFRT-PKCα and pFRT-PKCθ together it was 80%. Forty-eight hours later, an equal number of transfected cells (1x 10^6 per sample) were stimulated with either PMA (10 ng/ml) or prostratin (10 μM). Twelve hours later, cells and supernatants were harvested and luciferase activities were measured and normalized as described above. (B) Induction of p24 Gag production by PMA and prostratin in latently infected J 1.1 T cells. p24 data are shown as the fold increase of p24 of either PMA- or prostratin-stimulated samples to control (dimethyl sulfoxide [DMSO])-treated samples. Levels of p24 from supernatants of J1.1 cells were measured by using a p24 ELISA. p24 concentrations (in ng/ml) were normalized to *Renilla* luciferase light units. All experiments were performed at least three times in duplicate.

effect on HIV LTR transcription as suppression of both PKC isoforms together, suggesting that PKCα and PKCθ act in sequence to activate HIV LTR transcription. These findings are consistent with our previous observation that PKCα acts upstream of PKCθ to activate NF-κB following TCR ligation (54). Exactly how PKCα upregulates PKCθ is undefined, yet potential direct phosphorylation of PKCθ by PKCα at the plasma membrane may be required for either full PKCθ activation or to prolong PKCθ signaling following either CD3/CD28 engagement or prostratin stimulation. The murine PKCθ undergoes inducible phosphorylation at the C terminus on two adjacent (Thr-692 and Thr-703) residues following TCR or phorbol ester stimulation, which is required for NF-κB activation (52). We observed that the C-terminal phosphorylation of PKCθ occurs in a PKCα-dependent manner (data not shown), suggesting that PKCα can directly phosphorylate the two adjacent serine residues, S685 and S703, in human PKCθ (unpublished data). Therefore, PKCα-mediated phosphorylation prolongs PKCθ activation which, as we have previously demonstrated (53), is required for efficient NF-κB transcription. Consequently, persistent PKCθ activation may be necessary for efficient HIV LTR transcription and subsequent HIV reactivation from latency. This contention is supported by the fact that the constitutively active form of PKCθ provides prolonged signaling and, therefore, it induces HIV LTR transcription in primary $CD4^+$ T cells (Fig. 1C) and efficiently reactivates HIV from latency (Fig. 3D). Finally, we demonstrated that suppression of either PKCα or PKCθ inhibits prostratin-mediated or CD3/CD28-mediated HIV LTR transcription via κB sites. However, suppression of PKCθ also has a significant

effect on prostratin-induced HIV LTR (ΔκB) transcription and a smaller effect on CD3/CD28-induced HIV LTR (ΔκB) transcription, potentially due to inhibition of other transcription factors, such as AP-1. Previously it was shown that prostratin induces both NF-κB- and AP-1-dependent transcription (57). Our data are in agreement, since constitutively active forms of both PKCα and PKCθ can induce transcription of HIV LTR lacking κB sites (Fig. 3B) and potently induce AP-1-dependent transcription in J1.1 T cells (data not shown). However, it is noteworthy that the magnitude of AP-1 activation by PKCθ is much greater than that by PKCα. Therefore, selective inhibition of CD3/CD28- and prostratin-induced HIV LTR (ΔκB) transcription by suppression of PKCθ but not PKCα indicates that PKCα and PKCθ pathways are divergent. This is in agreement with the previous observation that PKCα activates NF-κB, but not AP-1, in a PKCθ-dependent manner (54). It is possible that the divergence in the PKCα and PKCθ signaling pathway occurs on the level of Ste20-related proline- and alanine-rich kinase, which recently has been shown to mediate PKCθ, but not PKCα, signaling to AP-1 (34). Therefore, while both PKC isoforms induce HIV LTR transcription via κB sites, PKCθ also contributes in HIV LTR transcription via AP-1 sites. The conclusion that both PKCα and PKCθ are required for HIV reactivation is in agreement with recently published data demonstrating that CD3/CD28 costimulation and prostratin treatment reactivate HIV from latency in the SCID-HU Thy/Liv model (9) in both a PKC- and NF-κB-dependent manner. Similarly, a role for PKC in mediating prostratin-induced HIV reactivation is further supported by observations that prostratin induces translocation of several conventional, novel, and atypical PKC isoforms to the cell membrane, suggesting that all these PKC isoforms can contribute to HIV reactivation (57). That suggestion is supported by observations that simultaneous inhibition of conventional and novel PKC isoforms together completely blocks prostratin-induced HIV reactivation (9, 57). However, inhibition of conventional PKC isoforms or PKCθ with inhibitor TER14687, which blocks PKCθ translocation, demonstrated that these drugs failed to prevent prostratin-induced HIV reactivation from latency (47, 57). These data are in sharp contrast to our results obtained with specific suppression constructs (Fig. 5), where we showed that PKCθ suppression abrogates both prostratin- and CD3/CD28-induced HIV LTR reactivation. Whether the previous report succeeded in achieving complete inhibition of conventional PKC isoforms is untested. Similarly, although TER14687 has been shown to prevent PKCθ translocation to the membrane following OKT3 stimulation by interfering with PKCθ-Fyn interactions (47), it is not clear whether the PKCθ-Fyn interaction is required for prostratin-induced PKCθ translocation and activation. Therefore, direct suppression of PKCθ with shRNA seems to be a more reliable approach to address the requirement for PKCθ function in prostratin-induced HIV reactivation. In doing so, we have demonstrated the absolute requirement for PKCθ in both TCR- and prostratin-induced HIV reactivation.

The sequential nature of PKCα and PKCθ activation is probably not limited to TCR or prostratin signaling pathways. Studies with another non-tumor-promoting phorbol ester, 12-deoxyphorbol 13-phenylacetate (DPP), suggest that DPP induces the same set of genes as prostratin and PMA (8), implying a similar PKCα- and PKCθ-dependent signal transduction pathway. In contrast to phorbol esters, synthetic diacylglycerol (DAG) lactones can reactivate HIV with the same efficiency as DPP without causing T-cell proliferation and upregulation of tumor necrosis factor alpha (24). Of note, these DAG lactones have 20- to 100-fold lower PKCα binding affinity than DPP, raising the possibility that DAG lactones can preferentially target PKCθ, thereby avoiding PKCα-mediated T-cell activation.

Altogether our data provide novel insights on the role of PKCα and PKCθ in HIV reactivation in T cells. The inability to efficiently reactivate HIV from latency represents a major barrier to HIV eradication. Identification of PKCα and PKCθ as necessary for HIV reactivation following TCR and phorbol ester stimulation indicates that these cellular targets may provide a key for the development of new therapies. Moreover, since PKCα activation has been implicated in tumorigenesis (reviewed in reference 39), selection of PKCθ as a therapeutic target may yield clinically relevant new agents.

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